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Tissue-specific expression of glucokinase: Identification of the gene product in liver and pancreatic islets

(hexokinase/immunoblotting/two-dimensional gel electrophoresis)

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ABSTRACT **The tissue distribution of glucokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was examined by protein blotting analysis. Antibodies raised against rat liver glucokinase recognized a single protein subunit with an apparent M_r of 56,500 on nitrocellulose blots of cytosol protein from liver, separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. A protein of identical electrophoretic mobility was detected by immunoblotting of cytosol protein from pancreatic islets. Hepatic glucokinase and the immunoreactive islet product bound to and were eluted from DEAE-cellulose at the same ionic strength. Glucokinase was displayed as a set of two spots with apparent pI values of 5.54 and 5.64 by immunoblotting after two-dimensional gel electrophoresis. The two isoforms appeared equally abundant in liver extract, whereas the component with a pI of 5.64 was predominant in islets. By quantitative immunoblotting, glucokinase was estimated to represent 0.1% of total cytosol protein in liver and 1/20th as much in islets. The glucokinase activity of both liver and islet cytosols was suppressed by the antibodies to hepatic glucokinase. Immunoblotting of cytosol protein from intestinal mucosa, exocrine pancreas, epididymal adipose tissue, kidney, brain, and spleen failed to reveal the glucokinase protein. Thus, significant expression of the glucokinase gene appears restricted to the liver and pancreatic islets.**

The mammalian hexokinases (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), which catalyze the first reaction of glucose metabolism, include four isoenzymes with distinct kinetic properties and tissue distribution (1). Glucokinase (or hexokinase type IV), the principal isoenzyme in liver, has a K_m for glucose of about 6×10^{-3} M, whereas the other three types of hexokinase have K_m values at least one order of magnitude lower (2, 3). Since the K_m of glucokinase lies within the normal range of intracellular glucose concentration in the liver, physiological changes in glucose concentration will cause parallel changes in the rate of glucose phosphorylation in that tissue. Moreover, adaptive changes in glucokinase amount are known to occur in liver under various hormonal and nutritional conditions (4). Therefore, glucokinase is regarded as an important regulatory enzyme of hepatic carbohydrate metabolism (5).

The expression of the glucokinase gene seems to be typical of the fully differentiated hepatocyte. Glucokinase is not detectable in fetal liver nor in poorly differentiated hepatomas (6, 7). Tissues other than the liver have been reported to contain a high-K_m glucose 6-phosphotransferase activity, but this activity was later ascribed to *N*-acetyl-D-glucosamine kinase (ATP:2-acetamido-2-deoxy-D-glucose 6-phosphotransferase, EC 2.7.1.59) rather than to glucokinase (8, 9). However, pancreatic islets of Langerhans do contain an enzyme with kinetic properties and chromatographic behavior indistinguishable from those of hepatic glucokinase (10). Indeed, Meglasson and Matschinsky have proposed that glucokinase might be the structural and functional equivalent of the "glucose sensor" of the insulin-producing beta cell (11).

In this paper we have sought to provide more direct evidence on the tissue-specific expression of the glucokinase gene by protein blotting analysis (12). First, glucokinase was identified by immunoblotting after $NaDodSO_4$/PAGE or two-dimensional gel electrophoresis of total protein from liver. A number of other tissues, including pancreatic islets, were then surveyed for the possible expression of the same gene product.

MATERIALS AND METHODS

Animals. Male Wistar rats, 180–250 g, were fed ad libitum. Drinking water was replaced by a 20% (wt/vol) glucose solution 20 hr before the experiments. The rats were killed by decapitation.

Preparation of Tissue Extracts. The tissues were excised and washed in ice-cold phosphate-buffered saline (10 mM sodium phosphate, pH 7.4/140 mM NaCl). All subsequent manipulations were done at 4°C. Homogenates were prepared in Teflon/glass homogenizers in 2 vol of homogenization buffer per vol of tissue. The homogenization buffer contained 20 mM Tris·HCl (pH 7.0), 80 mM NaCl, 4 mM $MgSO_4$, 5 mM EDTA, 2 mM dithiothreitol, and 20 mM glucose. The homogenates were centrifuged at $92,600 \times g_{max}$ for 1 hr to obtain clear cytosols. Samples of cytosol were frozen in liquid nitrogen, stored at −75°C, and lyophilized just prior to electrophoresis. Protein concentrations were measured by the Bradford assay (13).

Isolation of Pancreatic Islets. Islets were isolated from pools of three pancreata by a published procedure (14), except that the isolation medium was buffered with 30 mM Hepes instead of bicarbonate and was supplemented with 100 units of penicillin and 100 μg of streptomycin per ml, 8.4 mM glucose, and 20 amino acids at concentrations found in rat plasma (15). Approximately 400 islets were collected within 90 min of decapitation. The islets were homogenized as described above in 150 μl of homogenization buffer, and cytosol was obtained by centrifugation at $149,000 \times g_{max}$ for 40 min.

Assay of Hexokinases. Enzyme activity was assayed by measuring the formation of NADH in a coupled assay system containing glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim). The reduced coenzyme was measured fluorometrically. The assay conditions were essentially as described (16) except that all volumes were scaled up 2-fold. Enzyme assay was performed with cytosols prepared as described above except that the homogenization buffer contained 150 mM KCl instead of NaCl and was supplemented with 0.2% bovine serum albumin. Parallel incubations were conducted in the

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presence of 0.5 mM glucose (for assay of hexokinases types I, II, and III) and 100 mM glucose (for assay of total hexokinase). Glucokinase activity was calculated by subtraction.

Purification of Glucokinase and Production of Antibodies. Glucokinase was purified from rat liver by a published procedure (17). The protein was estimated to be more than 90% pure by $NaDodSO_4$/PAGE and was used for immunization of a sheep by a standard protocol. For partial purification of glucokinase in small cytosol samples, cytosol was applied batchwise to DEAE-cellulose preequilibrated in a buffer (20 mM Tris·HCl, pH 6.75/1 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol) containing 80 mM NaCl. After thorough washing in the same buffer containing 0.1 M NaCl, bound protein was eluted in buffer containing 0.375 M NaCl. Hepatic glucokinase was enriched about 10-fold by this procedure, with a yield of 60%.

Gel Electrophoresis and Immunoblotting. Lyophilized tissue extracts were dissolved in appropriate sample buffers, and proteins were separated by $NaDodSO_4$/PAGE (18) or high-resolution two-dimensional gel electrophoresis (19). Electroblotting of the resolved proteins on nitrocellulose filters was performed in 25 mM Tris/192 mM glycine/20% (vol/vol) methanol at 60 V for 4.5 hr. Purified glucokinase was transferred quantitatively under these conditions. After transfer, the remaining protein binding sites on the filters were blocked by incubation for 2 hr at room temperature in 20 mM Tris·HCl, pH 7.5/500 mM NaCl (TN buffer) supplemented with 3% (wt/vol) gelatin. Immunochemical detection of glucokinase was then accomplished by sequential incubations of the filters in the presence of the following reagents: (*i*) sheep antiserum against rat liver glucokinase; (*ii*) rabbit antibodies against sheep IgG (Genofit, Geneva); and (*iii*) peroxidase-conjugated goat antibodies against rabbit IgG (Bio-Rad). Antiserum and antibodies were diluted 1:300, 1:1500, and 1:3000, respectively, in TN buffer containing 1% gelatin and 0.3% Triton X-405. Incubation with antiserum to glucokinase was for 1 hr at room temperature and overnight at 4°C. The second and third incubations were for 2 hr at room temperature. The filters were washed in at least five changes of TN buffer after each incubation. The peroxidase-antibody conjugate was revealed by reaction with 4-chloro-1-naphthol and H_2O_2 as substrates (20).

RESULTS

Immunoblotting of Rat Liver Glucokinase. A sheep antiserum was tested for the presence of specific antibodies against glucokinase by probing a nitrocellulose blot of total liver cytosol proteins separated by $NaDodSO_4$/PAGE. A single major band corresponding to a protein of apparent M_r 56,500 was stained (Fig. 1, lane 4). This band was absent when preimmune sheep serum, or antiserum that was titrated with an excess of homogenously pure glucokinase prior to immunoblotting, was used (not shown). Therefore, we conclude that the present antiserum contains specific antibodies to liver glucokinase and can be used to detect this enzyme in complex mixtures of protein. The molecular weight estimate deduced from this and other similar experiments is slightly larger than earlier values (48,000–52,000) determined after enzyme purification (17).

The stained glucokinase band in the cytosol lane (Fig. 1, lane 4) can be compared with signals produced by graded amounts of pure glucokinase (Fig. 1, lanes 1–3) to evaluate the relative abundance of the glucokinase protein in liver. The band displayed after electrophoresis of 2 μg of total cytosol protein appears slightly fainter than the 3-ng glucokinase standard. This suggests that glucokinase represents approximately 0.1% by mass of total cytosol protein in rat liver during glucose feeding (i.e., in the induced state). It

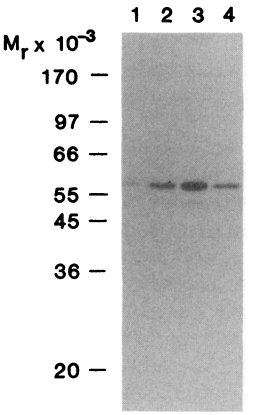

FIG. 1. $NaDodSO_4$/PAGE and immunoblot of total cytosol protein and purified glucokinase from rat liver. Gel electrophoresis, protein blotting to nitrocellulose, and immunoanalysis were done as described. Lanes: 1–3, 1, 3, and 9 ng of pure glucokinase; 4, 2 μg of total cytosol protein from the liver of glucose-fed rat.

is interesting to compare this estimate with a calculation based on published purification data. The highest specific activity reported for homogenous glucokinase is 150 units/mg of protein (17). The specific activity of glucokinase in cytosol in the present experiments was 0.07 unit/mg of protein. Therefore, the theoretical purification factor would be 2150-fold (150:0.07), in broad agreement with the immunoblotting data.

Pancreatic Islets Contain a Protein Immunologically Related to Hepatic Glucokinase. An enzyme activity similar to glucokinase as regards hexose substrate specificity, K_m for glucose, and binding to affinity or ion-exchange chromatography resins has been described in pancreatic islets (10, 16), suggesting that the glucokinase gene might be expressed in islets as well as liver. The availability of an antibody probe for the gene product allowed us to address this question directly. In an immunoblot of islet cytosol protein resolved by $NaDodSO_4$/PAGE (Fig. 2*A*), an islet protein (lane 4) with the same electrophoretic mobility as liver glucokinase (lanes 1–3) reacted with the antibody. The relative abundance of this

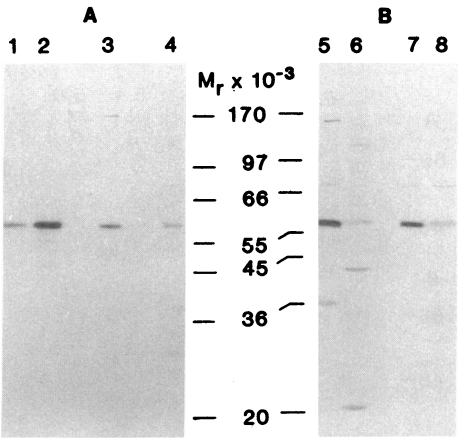

FIG. 2. Immunoblots of cytosol proteins from liver and pancreatic islets. Techniques for gel electrophoresis and immunoblotting are the same as in Fig. 1. (*A*) Immunoblot of purified liver glucokinase [lanes 1 (3 ng) and 2 (9 ng)] and of total cytosol protein from liver [lane 3 (1.5 μg)] and from pancreatic islets [lane 4 (14 μg)]. (*B*) Immunoblot of total cytosol protein from liver [lane 5 (4 μg)] and islets [lane 6 (42 μg)] and of DEAE-cellulose-eluted protein from liver [lane 7 (DEAE-cellulose eluate corresponding to 8 μg of applied cytosol protein)] and from islets [lane 8 (DEAE-cellulose eluate corresponding to 85 μg of applied cytosol protein)].

protein appeared about 20 times higher in liver than in islets, based on the protein content of the samples and the staining intensity of the specific band. This inference assumes similar transfer efficiency and immunoreactivity after transfer for the two samples.

Glucokinase can be partially purified from liver cytosol by DEAE-cellulose chromatography. We investigated the binding of the immunologically related islet protein to DEAE-cellulose. As shown in an immunoblot of crude cytosols and DEAE-cellulose-purified proteins (Fig. 2*B*), the glucokinase-related product present in islet cytosols (lane 6) bound to DEAE-cellulose and was eluted (lane 8) at the same ionic strength and with similar yield as was hepatic glucokinase (lanes 5 and 7). In this experiment, additional strong bands were visible after immunochemical staining, especially in lanes containing crude cytosols from islets and liver. These bands are abundant tissue proteins that are present in vast excess over glucokinase and that appeared to interact nonspecifically with antibodies. Such interactions were more or less pronounced in different experiments, without obvious reasons. Similar bands were also seen in control blots incubated with preimmune sheep serum.

Analysis of Glucokinase by Two-Dimensional Gel Electrophoresis. We further characterized hepatic glucokinase and the related protein of pancreatic islets by high-resolution two-dimensional gel electrophoresis followed by immunoblotting. In this system, liver glucokinase was displayed as a set of two spots with apparent pIs of 5.54 and 5.64 (Fig. 3*A*). The immunoreactive islet protein comigrated exactly with the pI = 5.64 isoform (Fig. 3*B*). In this and other experiments, the more acidic component was barely detectable in islets, whereas the two isoforms were about equally represented in liver cytosol. Although this data suggests a possible difference in isoform ratio between the two tissues, we cannot rule out an artifactual difference in isoelectric focusing due to overall differences in the composition of the tissue samples.

Suppression of Liver and Islet Glucokinase Activity by Antibodies. We tested the antibodies raised against hepatic glucokinase for their ability to suppress glucokinase activity in islet cytosol. When fixed volumes of cytosol from liver or islets were titrated with increasing volumes of antiserum, glucokinase activity was inhibited in a dose-dependent manner (Fig. 4). We consistently noted, however, that more antibody was required for neutralization of a given amount of glucokinase from islets than from liver. It is unclear whether

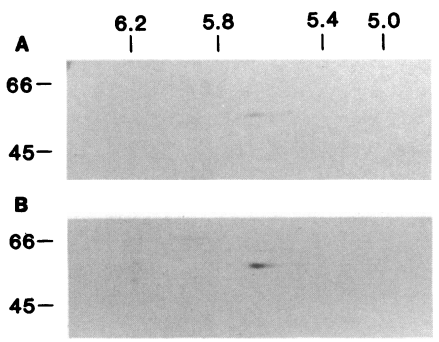

FIG. 3. Two-dimensional gel electrophoresis and immunoblots of total cytosol protein from liver and pancreatic islets. Electrophoresis, protein blotting, and immunoanalysis of glucokinase were done as described. (*A*) Immunoblot of 2.5 μg of liver cytosol protein. (*B*) Immunoblot of a mixture of 2.5 μg of liver cytosol protein and 36 μg of islet cytosol protein. Radioactive [^{14}C]methylated albumin was added to the samples prior to electrophoresis to serve as an independent benchmark protein, and autoradiograms of the blots were used to align *A* and *B*. The blots cover a molecular mass range from 88,000 to 35,000 daltons. The numbers in the upper line define the pH gradient in the isoelectric focusing dimension.

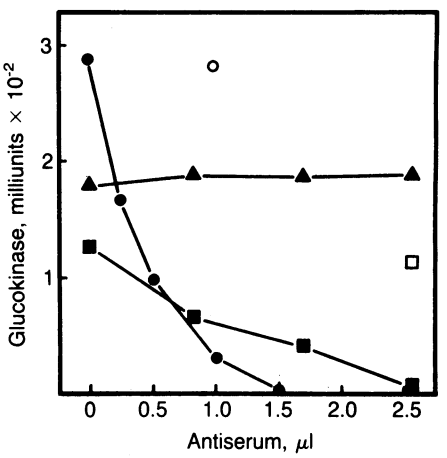

FIG. 4. Immunotitration of glucokinase. Samples of cytosol from liver (●) and from pancreatic islets (■) containing constant amounts of protein were incubated with increasing concentrations of anti-glucokinase antiserum for 30 min at 37°C and 1 hr at 4°C prior to assay of hexokinase activity. The enzyme assay was performed as indicated. Glucokinase activity in samples incubated without antiserum was the same as in nonincubated samples. ▲, Apparent "glucokinase" activity of samples of adipose tissue cytosol incubated with antiserum; ○ and □, control samples of liver and islet cytosol incubated with preimmune serum.

this difference is due to a lesser affinity of the antibodies for the islet enzyme and/or a reduced catalytic efficiency of this enzyme, or whether it reflects a nonspecific impairment of the antigen–antibody reaction in islet cytosol. Assayable glucokinase from liver or islets was not inhibited by control preimmune serum. Moreover, the high-K_m glucose 6-phosphotransferase activity of adipose tissue, which does not correspond to authentic glucokinase (see immunoblotting data below), was unaffected by the antiserum.

Glucokinase Is Not Detectable in Other Tissues. The final set of experiments examined the tissue distribution of glucokinase. Soluble proteins from a number of tissues were subjected to NaDodSO$_4$/PAGE and immunoblotting (Fig. 5). Glucokinase was undetectable in tissues other than liver and

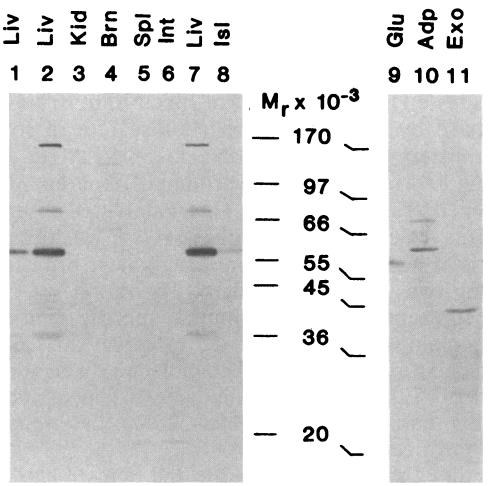

FIG. 5. NaDodSO$_4$/PAGE and immunoblot of total cytosol protein from various tissues. Gel electrophoresis and immunoblotting were done as indicated in Fig. 1. Tissues analyzed are: liver (Liv), kidney (Kid), brain (Brn), spleen (Spl), small intestine mucosa (Int), pancreatic islets (Isl), epididymal adipose tissue (Adp), and exocrine pancreas (Exo). Samples contained 20 μg of total cytosol protein except the liver sample in lane 1 (2 μg). The lane "Glu" contained 3 ng of purified glucokinase.

endocrine pancreas. On the other hand, unspecific bands corresponding to abundant tissue proteins were visible, particularly in adipose tissue and exocrine pancreas extracts. Of particular note is the absence of enzyme protein in intestinal mucosa and adipose tissue, since both of these tissues exhibit substantial high-K_m glucose 6-phosphotransferase activity in standard enzymatic assays (refs. 8 and 21; unpublished observations). Thus, the present data support the conclusion that this activity does not reflect the presence of glucokinase but should be attributed to other enzymes, such as *N*-acetyl-D-glucosamine kinase and/or hexokinase type II.

DISCUSSION

The results presented here show that pancreatic islets contain a protein closely related or identical to the hepatic enzyme glucokinase. First, both the liver enzyme and its counterpart in islets have a M_r of 56,500, as deduced from NaDodSO$_4$/PAGE. Second, both proteins are displayed as sets of two spots in two-dimensional electrophoresis gels, with apparent pIs of 5.54 and 5.64. Third, and most important, the islet product is immunologically related to hepatic glucokinase. In fact, the preeminent criterion for its identification was recognition by specific antibodies directed against hepatic glucokinase. These antibodies were able to suppress glucokinase activity in islet cytosol, albeit with reduced potency as compared to the inhibition of glucokinase from liver. The reason for this discrepancy is unknown.

The charge heterogeneity of glucokinase revealed by two-dimensional gel electrophoresis is reminiscent of earlier evidence for distinct electrophoretic forms of the enzyme in starch or agarose gels (7, 9, 21). The biochemical basis for multiple isoforms of glucokinase is unknown. Microheterogeneity of charge is a rather common finding when proteins are analyzed by isoelectric focusing in the presence of urea and may result from technical artifacts (19, 22). Nevertheless, our data raise the intriguing question of possible post-translational modifications of glucokinase and, further, of the possible significance of multiple enzyme forms for the regulation of glucokinase activity. As noted above, the relative amounts of isoforms appear to differ in liver and islets. However, this difference should be interpreted with caution in view of the fact that heterogeneous samples from the two tissues were analyzed.

On the basis of immunoblotting data, glucokinase is estimated to constitute about 1:1000 by mass of total soluble protein in liver and 1:20,000 in the endocrine pancreas. The enzyme was undetectable in all of the other major tissues examined, in spite of a detection limit in the order of 1:100,000 of total soluble protein.

In conclusion, this study strongly suggests that the glucokinase gene is expressed to a significant degree only in liver and pancreatic islets. Glucokinase occupies a key position in hepatic glucose metabolism (5). According to the hypothesis of Matschinsky and colleagues (11), glucokinase would subserve an equally important function in the pancreatic islet, where it would act as the glucose-"sensing device" of the insulin-producing beta cell. Whether glucokinase expression is a distinctive feature of the beta cell as opposed to the other cell types of the islet has not been determined. However, there is circumstantial evidence to support this view. First, beta cells constitute the bulk of the islet mass. Second, an enzyme similar to liver glucokinase has been partially purified from rat insulinomas (23), which contain mostly tumoral beta cells (24). Finally, we have recently identified glucokinase in such tumors by immunoblotting.

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